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Comparison of urinary aflatoxin M1 and aflatoxin albumin adducts as biomarkers for assessing aflatoxin exposure in Tanzanian children

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Abbreviations: [**AFM₁,** aflatoxin M_1; **AF-alb,** aflatoxin albumin adduct; **AFB₁,** aflatoxin B_1]

Abstract

Purpose: To determine levels of urinary AFM1 in children and correlate the concentrations with previously reported aflatoxin albumin adduct (AF-alb) levels in these children.

Materials and Methods: Matched urine and blood samples were collected from 84 Tanzanian children aged 6-14 months old. From 31 children in one village (Kigwa), samples were collected at three time points six months apart. Samples were collected from 31 and 22 children from two different regions at the second time point only. Urinary AFM_1 was measured using a commercial ELISA kit with a modified protocol to improve sensitivity. AF-alb was measured using an established ELISA method.

Results: The relative ranking of the three villages for exposure to aflatoxin based on either AFM_1 or AF-alb biomarker measurements was the same. In Kigwa village, both AFM_1 and AF-alb levels were higher at six months post-harvest compared to baseline. However, at the next visit the AFM_1 levels dropped from a GM (interquartile range) of 71.0 (44.7, 112.6) at visit two to 49.3 (31.5, 77.3) pg/ml urine, whereas AF-alb levels increased from 47.3 (29.7, 75.2) to 52.7 (35.4, 78.3) pg/mg albumin between these two visits, reflecting the fact that AFM_1 measures short term exposure whereas AF-alb measures longer term exposure. There was a correlation between AFB1 intake and AFM1 excretion (r = 0.442, p = < 0.001).

Conclusions: Urinary AFM_1 is a good biomarker for AFB_1 exposure in Tanzanian children, reflecting geographical and temporal variations in exposure to this food borne toxin.

Introduction

Aflatoxins are a group of fungal toxins produced by Aspergillus flavus and Aspergillus parasiticus in a variety of food crops either before harvest or during postharvest storage (IARC, 2002; Pitt et al, 2013), with aflatoxin B1 (AFB_1) being the most common and most toxic. Epidemiological studies have demonstrated a correlation between chronic exposure to aflatoxins and increasing risk of primary liver cancer (IARC, 2002). Moreover, chronic exposure to aflatoxin has been linked to child growth impairment (Gong et al, 2002; 2004) and immune suppression (Turner et al, 2003; Jiang et al, 2008). Approximately 1 million child deaths each year have been attributed to child stunting, wasting and micronutrient deficiencies (Black et al, 2013) and aflatoxin may be contributing to this toll, as aflatoxin contamination of weaning food made from maize and groundnuts frequently affects this vulnerable group in sub-Saharan Africa.

When food contaminated with aflatoxin is consumed by humans, part of the ingested toxin is metabolised by the liver to form metabolites that can be detected in human biological samples, including aflatoxin albumin adduct (AF-alb) in blood, aflatoxin N7 guanine in urine, and aflatoxin M1 (AFM_1) in urine or in breast milk. These metabolites can also serve as biomarkers of aflatoxin exposure (Routledge and Gong, 2011). One of the most frequently used biomarkers of aflatoxin exposure is AF-alb, which represents exposure over the past 2-3 months, based on albumin half-life (Chapot and Wild, 1991). Previous studies in which this biomarker has been applied have shown that aflatoxin exposure is common in some African populations and is associated with adverse health outcomes including deaths due to acute poisoning (Wild and Gong, 2010). AFM_1 can be detected in urine and has served as a biomarker of recent (24 hour) exposure to aflatoxin in adult populations for many years (Zhu et al, 1987; Groopman et al, 1992). However, few studies have assessed AFM_1 in children's urine and the relationship between the levels of AFM1 and AF-alb in children has not yet been established.

We have previously published results from a study of dietary exposure of Tanzanian children to mycotoxins, in which AF-alb was used to assess exposure to aflatoxin (Shirima et al, 2013; 2015;

Routledge et al, 2014). The urine samples collected from the children during the previous study provides an opportunity to analyse urinary AFM1, using an optimised commercial direct ELISA kit, and compare AFM_1 and AF-alb biomarkers of aflatoxin exposure in young children.

Clinical Significance

The measurement of AFM1 as a marker of aflatoxin exposure may benefit molecular epidemiology studies of the impact of aflatoxin on health.

Materials and methods

Subjects and sample collection

Urine and blood samples were collected from a cohort of children aged 6-14 months old (n =84). The children were from three villages; Kigwa, Nyabula and Kikelelwa in Tabora, Iringa and Kilimanjaro regions in Tanzania, respectively. Ethical approval was obtained from the National Institute of Medical Research in Tanzania and the University of Leeds, United Kingdom (HSLT/09/005), following review of the human subjects research proposal. Informed written consent was obtained from the mothers or children's care takers. Out of the 84 studied children, 31 were from Kigwa village (Kilimanjaro region), and their urine samples were collected at a series of three visits; at maize harvest season (July 2010), six months later (January 2011), then finally at the new maize harvest season (July 2011). In other children (31 from Nyabula village in Iringa region and 22 from Kikelelwa village in Kilimanjaro region), the urine samples available for analysis were those which were collected at one survey point only (six months after the harvest in January 2011) as opposed to Kigwa village in which the urine samples were from all three survey points.

Maize intake and AFB_1 contamination levels

Maize intake for each child was calculated based on a 24-h dietary recall questionnaire administered at the visit when urine samples were collected (Shirima et al, 2015). Levels of AFB_1 contamination in dried maize porridge samples taken from each family at the same time were measured by a standard HPLC method (Stroka et al, 2000) in the Tanzania and Food Drug Authority laboratory as previously reported (Routledge et al, 2014).

Analysis of aflatoxin-albumin adducts in blood

The methodology for collection of blood samples from these children and the subsequent analysis of AF-alb is as previously reported (Shirima et al, 2013). Briefly, albumin was extracted from blood, digested, purified and AF-alb was detected by a competitive ELISA using a rabbit polyclonal antibody (Chapot and Wild 1991). The method detection limit was 3 pg/mg albumin. Only those AF-alb results for which matched urine collections were available are included in the present study.

Analysis of urinary AFM_1

The first morning urine sample from each child was collected by the mother or child caretaker on two consecutive days using paediatric urine bags. The urine samples were kept in a cold box immediately after collection and were then kept frozen at -80 ºC prior to analysis. Urinary AFM_1 as a biomarker of aflatoxin exposure was determined using the Helica AFM_1 ELISA kit (Helica Biosystem, Inc., Santa Ana, CA, USA), according to manufacturer's instructions but with some minor modifications. In brief, following urine centrifugation, 50 µl of the supernatant was mixed with the same volume of water before 200 µl assay buffer was mixed in. This is a modification of the manufacturer's instructions as the dilution of the sample here was 1:1 rather than 1:19. This allows for the detection of lower concentrations of $AFM1$ in the sample. 100 µl of the mixture was transferred onto a microplate pre-coated with a mouse anti-$AFM1$ monoclonal antibody to allow binding of AFM_1 in the sample to the antibody on the plate. This was followed by incubation with 100 µl of aflatoxin horseradish peroxide conjugate for 15 minutes to allow for binding of the conjugate to the remaining unbound coated antibody. After reaction of the conjugated enzyme with the substrate, colour was read at 450 nm using a plate reader. Each sample was tested in duplicate. A urine matrix standard was used for the calculation of the sample concentration. The limit of detection was 15 pg/ml, and the recovery ranged from 73-111%, which was in consistent with the manufacture's manual.

Two blank urine samples were spiked with AFM1 at 200 and 500 pg/ml to serve as Quality Controls (QCs) that were run alongside each batch of samples. Additionally, the QCs were tested with sequential dilution at 1:2, 1:5 and 1:20 in order to investigate the recovery.

Statistical analysis

Concentrations of AFM_1 and AF-alb were not normally distributed and therefore non-parametric tests were used for statistical analysis for tests which involved these parameters and data was presented as median and inter-quartile range. For the data analysis, samples with AFM_1 or AF-alb levels below the LOD were assigned half the value of the detection limit. Friedman and Wilcoxon signed-rank tests were used to investigate the seasonal differences between AFM_1 concentrations measured at various recruitment times, for the children from the Tabora region (n = 31). Non-parametric Spearman's test was used for all the correlation analyses.

A p-value of ≤ 0.05 was considered statistically significant except for ≤ 0.01 for Spearman's test. All statistical analyses were performed using the IBM SPSS Statistics for Windows version 20 (SPSS Inc, Chicago, IL, USA).

Results

Assay accuracy and precision

The determination of the accuracy and precision of the assay was by the analysis of two QCs with spiked concentration of 200 and 500 pg/ml in blank urine that were diluted to give a range of concentrations of AFM1 to be measured (see Table 1). As a measure of accuracy of the method, the recovery varied between 90% and 119% of the true value. The precision of the method was expressed as the coefficient of variance (CV%), which was never higher than 11%. Because the samples are only diluted 1 in 2, rather than 1 in 20 as per the manufacturer's instructions, the LOD is increased to

15 pg/ml. Table 1 shows that even 10 pg/ml in diluted spiked standards was detectable with good accuracy.

Levels of urinary AFM$_1$

AFM$_1$ was detected in 86% of samples tested at a level above LOD, ranging from 15 to 2840 pg/ml; geometric mean (GM) (95% confidence interval) 36.5 (30.8, 46.3) pg/ml. The GM urinary AFM$_1$, collected from Kigwa village (Tabora region) at the three survey points are shown in Table 2, along with AF-alb values from the same children. A significant difference in mean levels of AFM1 between the three survey points was observed (p = 0.024). At recruitment, AFM$_1$ was detected in 87% of the children, with a GM concentration of 42.5 pg/ml (95% CI: 27.2, 66.5). The prevalence of positive samples and GM concentration increased to 100% and 71.0 pg/ml (95% CI: 44.7, 112.6), respectively at 6 months after recruitment. At the final visit, the GM concentration of AFM$_1$ was 49.3 pg/ml (95% CI: 31.5, 77.3) with 87% of samples testing positive.

Geographical variation in levels of urinary AFM1

Results of urinary AFM$_1$ from the three studied villages as obtained based on urine samples from the second survey point are presented in Table 3. A significant difference in levels of urinary AFM$_1$ among villages was observed (p < 0.001). The GM AFM1 was the highest in Kigwa, Tabora region (71 pg/ml) and lowest in Kikelelwa, Kilimanjaro region (12.9 pg/ml), with intermediate levels in Nyabula, Iringa region (29.3 pg/ml). The proportion of samples with detectable levels of AFM$_1$ showed a similar hierarchy of exposure, with 100% in Kigwa, 90% in Nyabula, and 59% in Kikelelwa.

Correlation of urinary AFM$_1$ and AF-alb

When the levels of urinary AFM$_1$ and AF-alb from the study individuals were compared, a highly significant correlation (r = 0.468, p < 0.001) of AFM$_1$ with AF-alb was seen (Figure 1). There was a similar pattern of aflatoxin exposure across the three geographical regions for both biomarkers, with the ranking of exposure between villages being determined as Kigwa>Nyabula>Kikelelwa using

either AFM_1 or AF-alb. However, when comparing the levels of exposure across three time points in Kigwa village, there was a difference in the relative levels of biomarker between visits two and three, with AFM_1 GM (IQR) reducing from 71.0 (44.7, 112.6) pg/ml urine at visit two to 49.3 (31.5, 77.3) pg/ml urine, whereas AF-alb levels increased from 47.3 (29.7, 75.2) to 52.7 (35.4, 78.3) pg/mg albumin at visit three.

Correlation of urinary AFM_1 and AFB_1 in the consumed maize

AFM_1 levels were compared to previously reported food intake and AFB_1 contamination data for the families in this study (Routledge et al, 2014). Figure 2 shows that urinary AFM_1 also correlated with AFB_1 contamination levels measured in family maize samples ($r = 0.442$, $p < 0.001$).

Discussion

This is the first study to compare AFM_1 and AF-alb biomarker levels in samples collected in very young children from a longitudinal study. Whilst the detection of the AF-alb biomarker allows for integration of aflatoxin exposure over the previous 2-3 months (Skipper and Tannenbaum, 1990), urine collection is less invasive, which is of particular relevance when sampling biomarkers in young children. The urinary biomarker AFM_1 has previously been compared to AF-alb biomarker levels in adults in a cross sectional study (Gan et al, 1988) but potential differences in metabolism and excretion between adults and children require confirmation that the two biomarkers are comparable in children.

In this study, the GM for AFM_1 of 36.5 (30.8, 46.3) pg/ml is higher than the levels previously found in urine of children aged 2-4 years in Guinea (GM = 16.3 pg/ml) in which AFM1 was detected in urine from 32 out of 50 children (Polychronaki et al, 2008). In the same study, 4/50 samples from Egyptian children aged 1-2.5 years showed detectable levels of AFM1, with a GM of 5.5 pg/ml. Another study in Nigeria using a multibiomarker method reported a low percentage of children with detectable AFM1 (Ezekiel et al, 2014), but that method had a much higher limit of detection (150 pg/ml), making comparisons difficult.

In adults, a range of AFM1 in urine has been reported in several studies in countries where aflatoxin exposure is common. In 2006, Jolly et al reported mean levels of 1,800 pg/mg creatinine in adults in Ghana. More recently, Redzwan et al (2012) found mean levels of 23.4 pg/ml urine in 160 adults in Malaysia, Lei et al (2013) reported a GM of 50.3 pg/ml in 600 adults in China and Ali et al (2016) reported a mean of 80 pg/ml in 95 adults from Bangladesh.

The ELISA method used to detect AFM_1 in the current study is a minor modification of a commercially available kit ELISA, which makes the application of the method widely available. This ELISA kit was previously used in the study of exposure in Malaysian adults by Redzwan et al (2012) and more recently by Ali et al in Bangladesh (Ali et al, 2016). In the current study, the ELISA was validated after inclusion of dilution of the urine samples, to give a limit of detection of 15 pg/ml of urine. Although the change in dilution factor may have altered how the assay performs compared to the manufacturer's protocol, we have validated the accuracy and precision of the assay using quality controls of known concentration and find that the assay performs reliably. We have not validated the selectivity of the modified assay. The advantage of this method is the high throughput and low cost of ELISA compared to HPLC quantification. It can be argued that HPLC can give more accurate results if multiple metabolites of aflatoxin are present, due to cross reaction of the antibody in the ELISA. Nevertheless, AFM_1 is the most common metabolite of AFB_1 in urine (Groopman et al, 1992), so results are unlikely to be distorted by possible co-detection of other metabolites. Recently, it has been reported that the method can produce false positive results at low concentrations of AFM_1 (Schwartzbord et al, 2016), although that study used urine samples from Haiti, where exposure to aflatoxin was much lower than in Tanzania, and reported a limit of detection (200 pg/ml) that was much less sensitive than the LOD we report here (15 pg/ml). In addition, the relative exposure levels for children as measured by AFM_1 ELISA in our study are supported by the results from the AF-alb biomarker analysis in the same children.

Our results reflect the seasonal variation of aflatoxin contamination that has been reported elsewhere (Castelino et al, 2014). An increase in aflatoxin contamination occurs after storage due to increased fungal growth and toxin production (Hell et al, 2003), which was reflected in the AFM_1 results here, with levels higher at the second visit, when stored maize was being consumed. Levels of AFM_1 were

then lower at the third visit when newly harvested maize was being consumed. It can be seen from Table 2 that a similar post harvest increase in exposure was determined using the AF-alb biomarker, with a large increase between visit 1 and visit 2. However, the subsequent drop in AFM_1 seen at visit 3 was not seen for the AF-alb biomarker. This reflects the fact that the AF-alb biomarker integrates exposure over the preceding 2-3 months (due to the half-life of albumin in blood) rather than the short-term (24 hour) exposure measured by the urinary AFM_1 biomarker. Therefore, at visit 3, compared to the AF-alb, the AFM1 biomarker better reflects the recent aflatoxin intake from the newly harvested maize.

There is a similar pattern of AFM1 and AF-alb biomarker levels within the same village for all three villages from different regions of Tanzania, with both biomarkers showing that exposure was highest in Kigwa and lowest in Kikelelwa. We have previously reported that contamination of maize flour was much more common in Kigwa compared to the other regions (Geary et al, 2016). Such differences between regions reflect differences in climate and soil conditions (Kimanya et al, 2008). The correlation we saw between AFM_1 levels and AFB_1 measured in maize flour is in agreement with an earlier study in China (Zhu et al, 1987; Gan et al, 1988), in adults.

Conclusions

In this study, a commercial ELISA kit protocol was modified to optimise detection of AFM_1. Application of this method to samples from a cohort of children in Tanzania shows that urinary AFM_1 is a good biomarker of recent exposure to aflatoxin in children, and that this biomarker correlates well with the AF-alb biomarker in children. Collection of urine is less invasive than collection of blood and the availability of the ELISA kit means that this method may be widely applicable in developing countries.

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Author contributions

GC carried out laboratory analyses and prepared the first draft of the manuscript; MEK led Tanzania fieldwork and contributed to study design; CPS oversaw sample collection; YYG carried out data analysis; MNR and YYG designed the study and wrote the manuscript.

Table 1. Accuracy and Precision of assay

AFM1 Concentration (pg/ml)[1]	Measured AFM1 (pg/ml) Mean ± SD[2]	CV %
10	10.3 ± 1.0	9
25	29.7 ± 3.0	10
40	47.0 ± 5.5	11
100	106.8 ± 11.6	11
250	223.5 ± 21.5	9

[1]25, 100 and 250 pg/ml concentrations were diluted from a 500 pg/ml spiked urine sample
10 and 40 pg/ml concentrations were diluted from a 200 pg/ml spiked urine sample
[2] n = 3

Table 2. Prevalence of detectable samples and geometric mean level (95% confidence interval) of AFM$_1$ (with range) and AF-alb in children at Kigwa village in Tabora Region by visit (sampling time point).

	Visit 1	Visit 2	Visit 3
AFM$_1$ detectable (%)	87	100	87
AFM$_1$ Geometric mean (95% CI)[1]	42.5 (27.2, 66.5)	71.0 (44.7, 112.6)	49.3 (31.5, 77.3)
AFM$_1$ Range[1]	ND[3] – 886	15.1 – 1950	ND – 2840
AF-alb detectable (%)	92	97	100
AF-alb geometric mean (95% CI)[2]	9.3 (6.6, 13.1)	47.3 (29.7, 75.2)	52.7 (35.4, 78.3)

[1]AFM$_1$, pg/ml urine; [2]AF-alb, pg/mg albumin; [3]ND, not detectable

Table 3. Prevalence of detectable samples and geometric mean level (95% confidence interval) of AFM$_1$ and AF-alb in children in three villages from different regions at visit 2.

Visit	Nyabula (Iringa)	Kikelelwa (Kilimanjaro)	Kigwa (Tabora)
AFM$_1$ detectable (%)	90	59	100
AFM$_1$ Geometric mean (95% CI)[1]	29.3 (22.1, 38.9)	12.9 (9.6, 17.5)	71.0 (44.7, 112.6)
AFM$_1$ Range[1]	ND[3] – 281	ND – 30.6	15.1 – 1950
AF-alb detectable (%)	97	36	97
AF-alb geometric mean (95% CI)[2]	12.4 (8.1, 18.9)	3.2 (2.3, 4.3)	47.3 (29.7, 75.2)
AF-alb Range	ND – 130.5	ND – 15	ND – 853.3

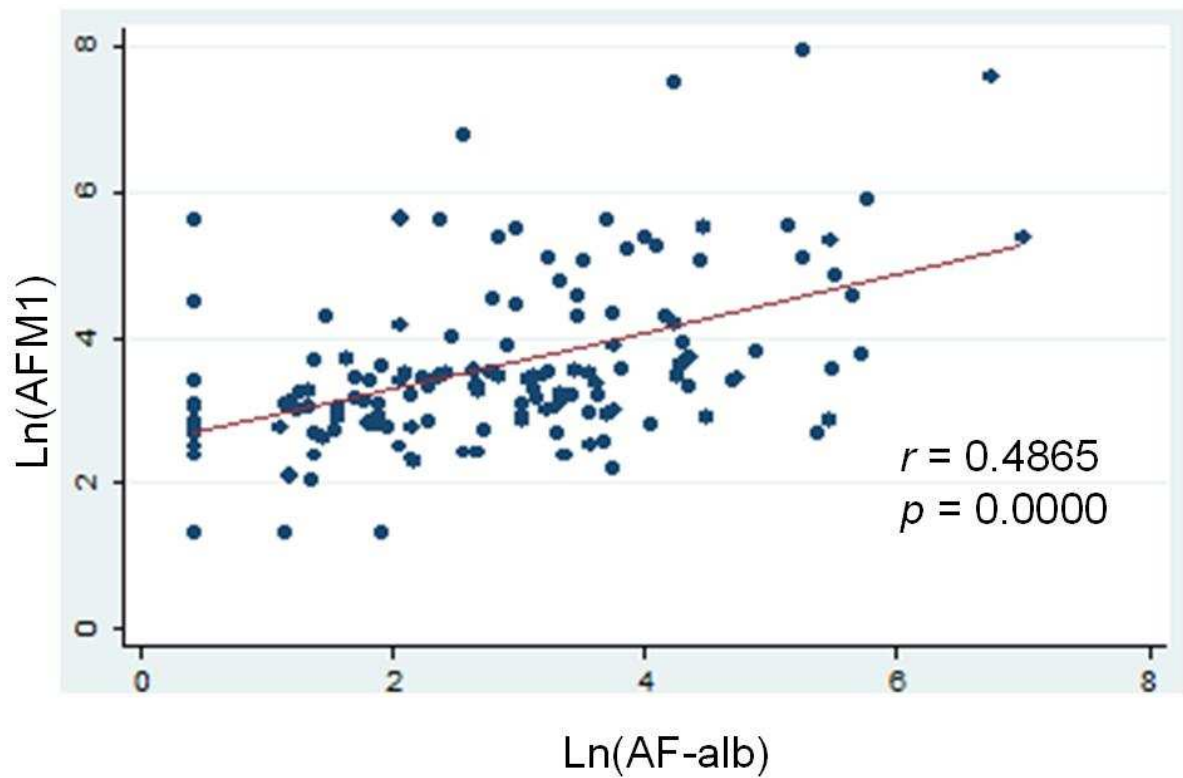[1]AFM$_1$, pg/ml urine; [2]AF-alb, pg/mg albumin; [3]ND, not detected.

Legends to figures

Figure 1. The correlation between urinary AFM_1 and blood AF-alb adducts (r =0.487, p <0.001). Both AFM1 and AF-alb adduct data are nature log transformed to obtain normal distribution.

Figure 2. The correlation between urinary AFM_1 and AFB_1 contamination levels in food (r =0.468, p <0.001). Both AFM_1 and AFB_1 data are nature log transformed to obtain normal distribution.

Figure 1. The correlation between urinary AFM$_1$ and blood AF-alb adducts (r =0.487, p <0.001). Both AFM1 and AF-alb adduct data are nature log transformed to obtain normal distribution.

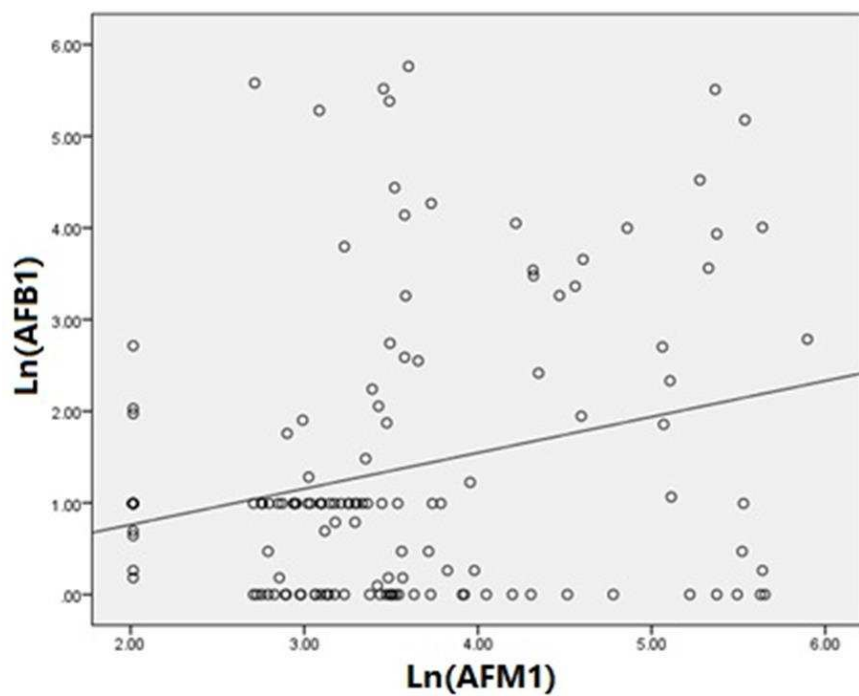Figure 2. The correlation between urinary AFM$_1$ and AFB$_1$ contamination levels in food (r =0.468, p <0.001). Both AFM$_1$ and AFB$_1$ data are nature log transformed to obtain normal distribution.